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Contents

The Circulatory Failure of Diphtheria. II. The Carbohydrate Metabolism in Diphtheria Intoxication. FRANCIS F. SCHWENTKER AND WILLIAM W. NOEL 259

A Study of Resorption from the Biliary Tract,—With Especial Reference to the Morphology and Permeability of the Cystic Epithelium. W. L. WINKENWERDER 272

Guanidine Base Concentration in Blood of Normal Individuals and in Patients with Liver Injury. READ ELLSWORTH 296

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THE CIRCULATORY FAILURE OF DIPHTHERIA

II. The Carbohydrate Metabolism in Diphtheria Intoxication

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The study of disease from the nutritional standpoint is interesting more and more workers in the field of investigative medicine. It is becoming apparent that toxemias of various kinds may cause profound metabolic disturbances. Thus, Hibbard and Morrissey (1) have reported the occurrence of transitory glycosuria in about one-fourth of the cases or nearly all of the fatal cases of diphtheria. Cartaginova (2) has called attention to the fact that in diphtheria a condition of hypoglycemia seems to parallel the seriousness of the disease. Lereboullet, Marie and Leprat (3) and later Lereboullet and Pierrot (4) have shown that in a considerable proportion of the grave cases of diphtheria definite hypoglycemia was present, while in the mild or moderate cases the blood sugar content remained unchanged.

Since dextrose is the precursor of glycogen and glycogen is the main nutritional basis for much of the body's activity, we felt that such disturbances in blood sugar might well reflect or be reflected by equivalent changes in the nutritional state of the tissues. We therefore undertook a study of the blood sugar values in experimental and clinical cases of diphtheria. Such studies have previously been made by other observers. Thus, several Japanese workers (5) (6) have reported hyperglycemia in fasting rabbits under the influence of diphtheria toxin, although Kuriyama (7) was unable to demonstrate in well-fed rabbits any marked change in the blood sugar. Mikami (8) injected large doses of toxin into the veins of rabbits and found that there was an immediate rise in sugar content of the blood, which reached a maximum in from 3 to 5 hours and was followed by a gradual reduction. When the quantity of toxin was so selected that

the rabbits lived over one day, not only was the hyperglycemia not seen, but in 24 hours after injection hypoglycemia could be observed. Rosenthal (9) also demonstrated a marked lowering of blood sugar values in animals which had been injected intravenously with large doses of diphtheria toxin. There is, therefore, no definite agreement in the results of workers in this field, but the varying nature of the conditions under which the experiments were done might well account for discrepancies.

In our study, 21 rabbits were injected subcutaneously with graded doses of diphtheria toxin, so that death occurred at varying times from 17 hours to 5½ days. The subcutaneous route for the administration of the toxin was chosen because it afforded slower absorption and would thus simulate clinical conditions more closely. The intravenous administration of the toxin, as practised by former workers, is open to criticism on this ground. Blood sugar determinations were then made by the micro-method of Hagedorn and Jensen (10) on blood allowed to flow from a cut ear vein. At times the blood pressure had fallen so low in moribund animals that it became necessary to resort to ventricular puncture to obtain blood specimens. Samples for analysis were taken every morning following an overnight fast. Food was allowed during the day. In the case of animals that had been given sufficient toxin to cause death within 24 hours, no food was allowed and frequent analyses were performed. When death was imminent, the rabbits were watched closely and, at the moment of death, blood was taken by ventricular puncture. In certain animals, the liver and the muscles of the hind-legs were removed and analyzed for glycogen (11).

Since the fluid loss of the body in diphtheria intoxications is known to be considerable, it seemed also desirable to follow roughly the relative changes in blood volume in these animals. This was accomplished by determination of the hemoglobin content of the blood at the same time that a sample was drawn for sugar analysis. Carefully standardized Sahli hemoglobinometers were used for this purpose.

Several interesting observations were made on these animals under the influence of diphtheria toxin. Rabbits dying within 48 hours after injection always died in violent convulsions lasting from 3 to 5 minutes. When death occurred after a longer time, it was more apt

TABLE I

HOURS AFTER TOXIN INJECTION	BLOOD SUGAR	HEMOGLOBIN	REMARKS
	Rabbit no. 3, female, weight 1940 grams		
	mgm. per 100 cc.	*per cent*	
0	141		Injection 0.041 cc. toxin per kilogram
24			Not ill
48			Very weak
49	110		Death
	Rabbit no. 4, female, weight 2490 grams		
0	133		Injection 0.028 cc. toxin per kilogram
25	119		
48			Appears ill
55	96		Diarrhea
58	104		Acutely ill
62	129		
63	125		
65	104		Death after several convulsions
	Rabbit no. 8, male, weight 1890 grams		
0	132	80	Injection 0.015 cc. toxin per kilogram
24	130	90	
48	135	105	
72	125	100	
83	141	100	Collapse. Diarrhea
84	118	100	Death after several convulsions
	Rabbit no. 9, male, weight 2220 grams		
0	97	80	Injection 0.01 cc. toxin per kilogram
24	143	80	
48	135	100	
72	106	100	
96	112	100	
120	140	100	
130			Definitely ill
133	138	95	Legs paralyzed. Collapse
135	159	95	Death
	Rabbit no. 10, female, weight 1550 grams		
0	111	65	Injection 0.026 cc. toxin per kilogram
17	139	65	
41	69	80	Moribund
42	82	100	Death in violent convulsions

TABLE I—*Continued*

HOURS AFTER TOXIN INJECTION	BLOOD SUGAR	HEMOGLOBIN	REMARKS
	Rabbit no. 12, male, weight 1900 grams		
	mgm. per 100 cc.	*per cent*	
0	113	80	Injection 0.52 cc. toxin per kilogram
1	110	80	
3	107	85	
5	99	85	
7	124	95	
9	110	100	Diarrhea
11	120	100	
15	42	120	Death after 10 minutes very violent convulsions
	Rabbit no. 14, female, weight 2000 grams		
0	125	75	Injection 0.01 cc. toxin per kilogram
22	110	75	
46	95	75	
70	194	90	Diarrhea
75	185	95	
77	138	100	Death, quietly Liver glycogen—0 Muscle glycogen—0
	Rabbit no. 17, male, weight 1700 grams		
0	140	95	Injection 0.294 cc. toxin per kilogram
19	226	110	
25	81		Death after convulsions Liver glycogen—0 Muscle glycogen—0.16 per cent
	Rabbit no. 18, female, weight 1330 grams		
0	117	75	Injection 0.012 cc. toxin per kilogram
24	116	80	
48	118	95	
72	122	90	
74	129	85	Death after few mild convulsions Liver glycogen—0 Muscle glycogen—0

TABLE I—*Concluded*

HOURS AFTER TOXIN INJECTION	BLOOD SUGAR	HEMOGLOBIN	REMARKS
Rabbit no. 19, female, weight 1770 grams			
	mgm. per 100 cc.	*per cent*	
0	115	75	Injection 0.011 cc. toxin per kilogram
24	119	75	
48	119	85	
72	154	80	
96	164	80	Slightly ill
120	166	75	
136	205	75	Death after few mild convulsions
Rabbit no. 20, female, weight 1580 grams			
0	135	75	Injection 0.10 cc. toxin per kilogram
24	148	90	
29	86	90	Death in convulsions

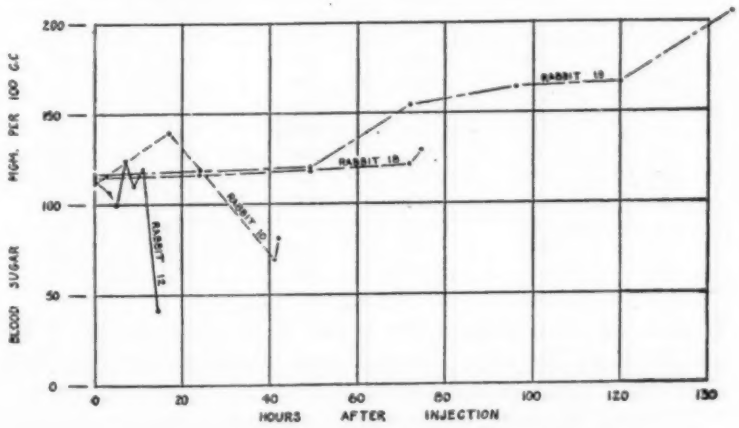

FIG. 1. BLOOD SUGAR CURVES OF FOUR RABBITS DYING FROM DIPHTHERIA INTOXICATIONS OF VARYING SEVERITIES

to be quiet and usually followed a period of several hours of unconsciousness. Towards the end of the intoxication food was refused; considerable water was ingested and a marked diarrhea, often bloody, was invariable.

In Table I are listed the findings in eleven of the cases. The remaining rabbits died at a time when no observer was present, so that blood specimens were not obtained at the death of the animal. The results are therefore omitted from this discussion.

Figure 1 is a graphic representation of four of the typical cases (Rabbits 10, 12, 18, 19) in which death occurred at varying times from 17 to 136 hours after the injection of the toxin. It will be noticed that in each there was a slight primary rise in blood sugar. Following this, there was a rapid fall in the early cases, but a further elevation where death occurred later in the intoxication. Thus, the terminal blood sugar value for rabbit 12 was 42 mgm. per cent, for rabbit 10, 82 mgm. per cent, while rabbits 18 and 19 showed elevations to 130 and 205 mgm. per cent. It would seem, therefore, that the blood sugar value increases as the severity of the intoxication decreases.

TABLE II

Relation between number of hours to death and per cent change in blood sugar

RABBIT NUMBER	HOURS TO DEATH	BLOOD SUGAR			PER CENT RISE OR FALL
		before	at death	difference	
12	15	113	42	−71	−63
17	25	140	81	−59	−42
20	29	135	86	−49	−36
10	42	111	82	−29	−26
3	49	141	110	−31	−22
4	66	133	104	−29	−22
18	75	117	129	+12	+9
14	77	125	138	+13	+10
8	84	132	118	−14	−10
9	135	97	159	+62	+64
19	136	115	205	+90	+78

Following this point, the per cent increase or decrease of the blood sugar value at death over the normal value before injection of the toxin was computed for each case. These are tabulated in Table II and have been plotted in Figure 2. Here, the per cent increase or decrease at death over the normal blood sugar value has been plotted against the hour of intoxication at which death occurred. A simple linear relation is evident. In other words, rabbits which die of diphtheria intoxication in less than 70 hours have blood sugar values less

than the normal figure; while those whose death occurs later than 70 hours show an increase over the normal. This increase becomes greater the later in the intoxication the rabbit dies; the hypoglycemia, however, is greatest in those animals dying soonest. The interpretation of this finding will be considered later.

The hemoglobin values are also significant. The animals which died of a severe toxemia (Rabbits 10, 12) showed a marked rise in

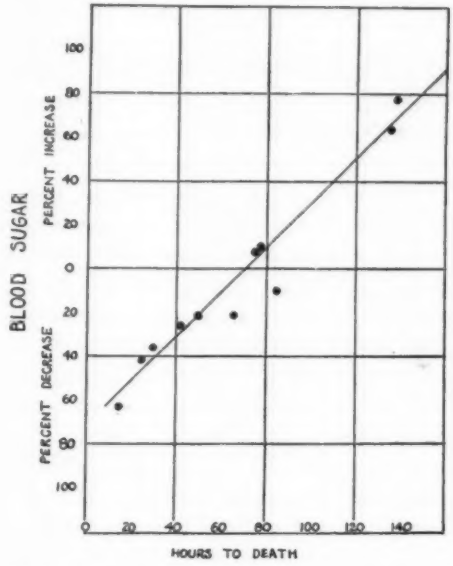

FIG. 2. SHOWING RELATION BETWEEN PER CENT INCREASE OR DECREASE OF BLOOD SUGAR VALUE AT DEATH OVER NORMAL BLOOD SUGAR AND HOUR OF INTOXICATION AT WHICH DEATH OCCURRED

hemoglobin values; when the toxemia was less severe (Rabbits 9, 16, 18, 19) the hemoglobin showed a primary rise in concentration with a subsequent fall. This increase in hemoglobin value is probably to be explained by a decrease in blood volume with concentration of the cellular elements. The later decreases are probably due to toxic blood destruction.

In three of the cases, the liver and muscles were analyzed for glycogen. Rabbit 17 died in the 25th hour of intoxication; 0.16 per cent glycogen was found in the muscles; there was none in the liver. In rabbits 18 and 14, dying at 15 and 77 hours, respectively, the liver and muscles were also entirely devoid of glycogen. This is in agreement with the results obtained by Rosenthal (9). In 4 rabbits dying of diphtheria intoxication at 11, 28, 29 and 29 hours he found the hepatic glycogen content to be 0, 0, 0, and 0.02 per cent, respec-

TABLE III

Blood sugar values following intravenous administration of dextrose

NUMBER	AGE	CONDITION	GIVEN INTRAVENOUSLY	BLOOD SUGAR				SUGAR IN URINE
				before	10 minutes	1 hour	2 hours	
	years							
11146	8	Normal	20 grams dextrose	98	138	81	95	0
11200	7	Normal	10 grams dextrose	80	98	84	91	0
11467	40	Normal	20 grams dextrose	76	150	100	89	0
11237	5	Diphtheria	10 grams dextrose	64	210	148	110	+
11284	4	Diphtheria	10 grams dextrose	102	230	Clotted		+
10881	12	Diphtheria	20 grams dextrose	72	224	158	148	
10863	7	Diphtheria	10 grams dextrose	139	298	182	148	+
11301	3	Diphtheria	10 grams dextrose	83	250	182	148	
11324	12	Diphtheria	20 grams dextrose 20 units insulin	98	235	52	106	+
11372	34	Diphtheria	20 grams dextrose 10 units insulin	93	190	68	89	+
11549	8	Diphtheria	20 grams dextrose 10 units insulin	66	303	83	78	

tively, while in the first three of these, the muscle glycogen was found to be only 0.165, 0.245 and 0.305 per cent. Two normal rabbits under identical experimental conditions showed 8.015 and 5.30 per cent glycogen in the liver.

We are able, therefore, to make quite definite conclusions that in diphtheria intoxication there is a marked disturbance in the carbohydrate metabolism. Rosenthal (9) in 1914 demonstrated that following the ingestion by diphtheria rabbits of 20 grams of dextrose,

the blood sugar rose to a definite hyperglycemia and that the return to normal came only after several hours. Similar observations have been made by Tisdall, Drake and Brown (12) on puppies and by Elkeles and Heimann (13) (14) (15) (16) and by Hector (17) (18) in clinical cases of diphtheria. Sweeney and Lackey (19) have studied this phenomenon in rabbits. They found that rabbits made toxic by diphtheria toxin have a definite decrease in tolerance for dextrose. This decrease becomes more marked during the course of the toxemia. There is, however, no noticeable effect of the toxemia on the gastro-intestinal absorption of the dextrose.

We have undertaken a further study along these lines. It was first attempted to study the changes in the blood sugar values of patients suffering from diphtheria after they had ingested the usual amounts of dextrose. However, the children often refused to coöperate and invariably vomited more or less of the dextrose, so that the procedure was finally abandoned and the intravenous route of administration was chosen instead. Blood samples for sugar analysis were obtained immediately before, at ten minutes, at one hour and at two hours following the intravenous injection of a 50 per cent solution of dextrose. In the smaller children 10 grams were administered in this fashion, the larger children receiving 20 grams. Control experiments were carried out on three normal persons. The method as described by Folin and Wu (20) was used for the blood sugar analyses.

In Table III have been grouped the results of these experiments. Normal children show very little change in blood sugar values after the injection of dextrose; none of the sugar is excreted in the urine. Patients suffering from diphtheria, on the other hand, show marked decrease in the ability to assimilate the dextrose from the blood stream. In each case, the blood sugar value after injection lay between 200 and 300 mgm. per cent, and had not yet returned to its original figure at the end of two hours. In every case tested there was an overflow of the dextrose into the urine according to Benedict's test (21). When, however, insulin is added to the injected dextrose, the blood sugar curves approach more nearly the normal. Even in these cases, however, the disturbed metabolism is indicated by the high sugar value at the end of 10 minutes. In Figure 3 have been plotted blood sugar curves characteristic of each type of experiment.

This ability of insulin to cause the assimilation of dextrose in the face of the disturbed carbohydrate tolerance of a diphtheria patient has also been noted by Sweeney (22). He found that dextrose tolerance tests on diphtheria rabbits gave results more nearly normal when insulin was injected 15 minutes after the ingestion of the dextrose. On the other hand, Lawrence and Buckley (23) have studied the blood sugar values in diphtheria rabbits following the injection of $\frac{1}{2}$–1

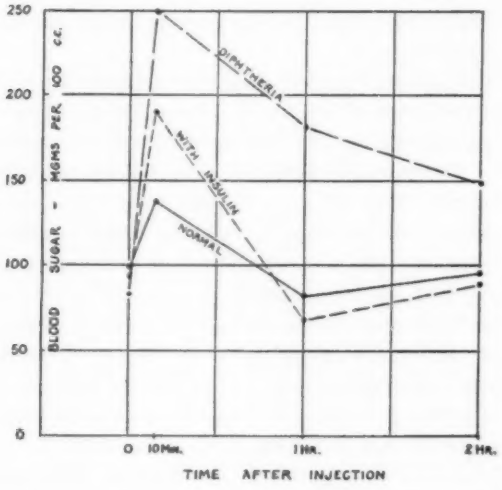

FIG. 3. BLOOD SUGAR CURVES FOLLOWING INTRAVENOUS INJECTION OF 20 GRAMS DEXTROSE INTO (1) NORMAL PERSON, (2) PATIENT SUFFERING FROM DIPHTHERIA, (3) DIPHTHERIA PATIENT, 10 UNITS OF INSULIN WERE ADDED TO THE DEXTROSE

unit of insulin and have found that there is some decrease in the usual hypoglycemic action of insulin in diphtheria intoxication.

We may sum up our evidence, then, as follows: 1. Severe diphtheria intoxication causes a marked pre-lethal hypoglycemia. In the less severe toxemias, so that death occurs later in the disease, the pre-lethal blood sugar value becomes progressively higher until after the third day a definite hyperglycemia becomes evident. 2. There is a marked reduction, indeed, in most cases a complete absence of hepatic

and muscular glycogen during the toxemia. 3. There is a definite inability to assimilate dextrose from the blood stream in diphtheria intoxication; this is progressive with the toxemia. 4. Insulin retains, to some extent, its power to cause assimilation of the dextrose in diphtheria intoxication.

We look, therefore, for an explanation of these phenomena. Lawrence and Buckley (23) drew their conclusions from a study of rabbits who died in 4 to 7 days after the toxin injection. They observed, therefore, only the rise in pre-lethal blood sugar value, and, linking this evidence with changes found in the adrenal and thyroid glands, have concluded that the delayed dextrose assimilation is due to a glycogenolysis produced by a stimulation of the "thyroid-adrenal" apparatus. Sweeney (22), while admitting that the progressive rise in blood sugar values in toxic animals is probably due to glycogenolysis, feels that the decreased tolerance to dextrose is better explained on the basis of a decreased glycogenesis. Rosenthal (9) has recognized both increased glycogenolysis and decreased glycogenesis.

Considering all of the data thus far presented, we are inclined to picture the mechanism in diphtheria intoxication as follows: In the first stages of the toxemia, there is an increase in the metabolic processes of the body; the glycogen stores in the liver and muscles are reduced to dextrose. This is evidenced by the increased blood sugar values in the early stages. Very probably, there is also an increased activity of the thyroid and adrenal glands. If the intoxication is severe, the glycogen and dextrose are rapidly consumed in the process and, when death supervenes, there is found a definite hypoglycemia and a complete absence of hepatic and muscle glycogen. If, however, the intoxication is of longer duration, there becomes apparent a progressive inability on the part of the body to assimilate carbohydrate. Most of this is probably due to a suppression of the production of insulin; some of the inability may also be due to a continued activity of the thyroid-adrenal apparatus. When this stage has been reached, there is a progressive rise in the blood sugar values (Figs. 1, 2), but the body is unable to utilize any of the dextrose. The absence of glycogen in the liver and muscles is evidence on this point. Naturally enough, when the body is no longer able to assimilate the glucose, nutritional disturbances are set up and cellular degeneration

occurs. The pathologists describe such lesions as a constant finding (24) (25) (26) and Warthin (27) has advanced the opinion that the fatty degenerative infiltration, cloudy swelling, and necrosis seen in the heart and voluntary muscles are due to nutritional disturbances. Josephs (28) has studied the metabolism of dogs following the injection of diphtheria toxin and demonstrated an increased nitrogen excretion which he feels comes largely from the muscular tissue. Fatty degeneration of the liver is also a common finding; this is to be expected when we consider that the depletion of the cells of their glycogen content must bring about the same marked nutritional disturbances as are present in the muscles. Warthin (27) has also demonstrated lesions in the bundle of His comparable to those in the myocardium. We feel sure that the muscular elements of the arteries and arterioles, were they examined, would also show equivalent changes. In fact, we believe that all the tissues of the body are affected by the diphtheria toxin and the resultant abnormal metabolism.

SUMMARY

We have presented evidence which points to a marked abnormality in the carbohydrate metabolism in cases of diphtheria intoxication. This is manifested by a primary increase in glycogenolysis in the first stage of the toxemia and a resultant rise in blood sugar. Following this there appears a marked hypoglycemia in very severe cases. In the more protracted cases, however, there seems to follow a decrease in glycogenesis, due probably to a suppression of the production of insulin, with a resultant hyperglycemia. The liver and muscles show a marked diminution in glycogen content in all cases.

We feel, therefore, that the symptoms of diphtheria intoxication are to a large part due to secondary nutritional disturbances in all of the essential body tissues.

The administration of insulin in these cases causes the assimilation of the dextrose, most probably through the usual glycogenic channels.

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A STUDY OF RESORPTION FROM THE BILIARY TRACT,— WITH ESPECIAL REFERENCE TO THE MORPHOLOGY AND PERMEABILITY OF THE CYSTIC EPITHELIUM[1]

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INTRODUCTION

Experimental investigation concerning the function of the gall-bladder has undergone a pronounced revival during the past decade. The time-honored hypothesis that the gall-bladder stores and concentrates the bile between periods of digestive activity and, in answer to hormonal stimulation, subsequently discharges its contents into the duodenum through contractions of its intrinsic musculature, has been severely questioned, and extensively reinvestigated. The most recent of these studies, particularly of Boyden (1925, 1926, 1928) and Ivy and Oldberg (1927–1928) strongly support the historic hypothesis. Boyden demonstrated, both in man and in cat, that specific food substances, notably egg-yolk, when ingested orally, elicit contractions and forthwith evacuation of the gall-bladder. Similar results were obtained by Ivy and Oldberg in experiments on the dog, in which the intravenous injection of highly purified, secretin-free extracts of the duodenal or jejunal mucosa, called by them cholecystokinin, was found to induce contractions and emptying of the biliary vesicle. However, certain investigators (Halpert, 1924, 1925, 1928; Sweet, 1924), discouraged by failures to prove the original hypothesis, have denied the expulsion of bile by the gall-bladder, and have proposed instead that all of the bile which enters this organ is eventually resorbed and returned to the body economy. Since the comparative chemical analysis of the hepatic and vesical bile by Hammersten (1893), with later confirmation by Brand (1903) and Rosenbloom (1913), it has been current knowledge that the gall-bladder possesses a striking power of concentrating its contents. These analyses reveal a marked percent-

[1] Submitted for publication April 18, 1929.

age increase of all biliary constituents with the exception of water and inorganic salts, which are contrariwise decreased. But the effort to promulgate and establish this resorbing activity as the sole function of the gall-bladder lacks complete scientific proof; for with the exception of the substances mentioned, no direct evidence has ever been adduced that, under normal physiological conditions, other constituents such as the bile pigments, the bile salts, cholesterin and lecithin, are resorbed through the gall-bladder wall. Sweet (1924) states that positive tests for bile pigments and acids were secured on the lymph withdrawn from the large, draining lymphatic trunks of the gall-bladder; but no descriptions of his experimental conditions and procedures were given, and no confirmatory work has subsequently appeared to substantiate his assertion.

The question of the resorption of neutral fat has received much attention. The frequent presence of this substance in the gall-bladder wall has often been interpreted as evidence of absorption. This view, first advanced by Virchow (1857), and later warmly maintained by Aschoff (1924), Boyd (1924) and Halpert (1924), has been supported by the more recent experimental results of several investigators (Igwanaga (1923), Kusnetzowsky (1924), Mentzner (1925), Demel and Brummelkamp (1924)). Each of these workers, employing practically identical experimental procedures, demonstrated that, following the replacement of the cystic bile by some form of neutral fat, there followed a definite increase in the stainable lipoids in the epithelial cells of the mucosa, in the delicate tunica subepithelialis, and in some instances, in the efferent lymphatics. In control experiments without the introduction of fat no such distribution of lipoids was observed. In other cases, the fat was injected into the common duct, prior to its ligation. After this procedure, an increase in the intracellular lipoids of the epithelial cells lining the larger hepatic ducts and the cystic and common duct was found. Therefore, an equivalent resorbing capacity was attributed to these cells, contrary to the conclusion previously reached by Rous and McMaster (1921).

In addition to evaluating numerically the concentrating power of the gall-bladder, Rous and McMaster suggested that the hepatic duct cells were excretory in function, because in prolonged experiments, in which the cystic and common ducts were ligated, so-called "white

bile" was produced. According to their observations, the stasis bile lost its characteristic color after a period of days, and became pale and limpid—a transformation which they considered was due to a secretion of fluid by the epithelial cells. Analyses of the stasis bile were not obtained during the first few hours of the experiment, but Brugsch and Horsters (1926) found in a subsequent study that concentration rather than dilution occurred, as evidenced by an increased pigment content and a heightened refractory index of the obstructed bile. The interpretation of Brugsch and Horster's findings, therefore, coincides with that of the investigators who demonstrated fat resorption by the epithelial cells of the duct system.

Another means of approaching the problem of absorption by the biliary tract consists of determining the ultimate fate of various foreign substances (for example, true solutions, colloidal dyes or particulate suspensions) introduced experimentally into the lumen of the gall-bladder. Following this procedure a number of investigators (Wertheimer and Lepage, 1896; Billard and Cavalié, 1900; Boyd, 1923; Igwanaga, 1923; Harer, Hargiss and Van Meter, 1922; Mentzer, 1925; Nakashima, 1926) have reported the recovery of foreign substances either in the bile, the liver, the urine, or in the draining lymphatics of the gall-bladder. Wertheimer and Lepage detected indigo-carmine sooner in the urine than in the thoracic duct lymph and therefore believed that resorption occurred more rapidly via the blood-vascular system. Billard and Cavalié showed that water, salt and potassium sulphocyanide were all resorbed and that the latter could be demonstrated qualitatively in the hepatic tissue immediately adjacent to the gall-bladder. Harer, Hargiss and Van Meter confirmed their experiment with potassium sulphocyanide by obtaining positive tests for it in the draining lymphatics. Employing Prussian blue reagents, Boyd observed histologically some of the Prussian blue in the epithelial cells of the mucosa and to a less extent in the subepithelial tissue spaces; but its presence either in the veins or in the lymphatics could not be detected. Following the injection of either particulate carmine, charcoal powder or India ink, Mentzer recovered these substances in the cystic lymph nodes. In spite of unconvincing experimental evidence he believed that cholesterin was also absorbed. Nakashima studied a series of dyes possessing various degrees of diffusibility and

concluded that the degree of diffusibility and not the chemical structure of the substance determined its absorption. Furthermore, observing that the dyes appeared much earlier in the bile than in the urine, he postulated a vesico-biliary lymphatic circulation. Finally, the experiments of Igwanaga, although unnoticed in the current literature, demand a brief discusion. He found that phenosulphonphthalein, methylene-blue, indigo-carmine and potassium iodide were all readily absorbed, appearing in the urine in twenty minutes on the average, and in the blood in fourteen minutes. With isotonic solutions the maximal excretion was reached at the end of the first hour; sixteen per cent and nine and one half per cent being eliminated, respectively, in rabbits and dogs. Dilution of these dyes, within limits, directly increased their excretion, the maximum being reached within the first hour, whereas concentration was followed by a latent period with the maximal excretion occurring during the second hour. Mechanical or bacterial inflammation augmented considerably the rate of resorption, the dyes appearing in the urine in from five to ten minutes. He concluded from these results that the biliary mucosa reacted essentially as a semipermeable membrane.

In the discussions and conclusions of the various experimental studies cited, emphasis has been placed on the lymphatic system as the important channel whence selected biliary substances and experimental solutions are returned to the general circulation. Nevertheless, several workers believe that the principal pathway of resorption takes place by the blood vascular system (Billard and Cavalié; Wertheimer, Igwanaga, Rous and McMaster and Mentzer). The demonstration of numerous lymphatic vessels in the gross specimen, as well as the frequent assertion that these channels are the chief means of disseminating infection, have undoubtedly encouraged the former conception. However, simultaneous analyses of the venous blood and of the lymph stream were not attempted in the series of experiments reviewed; and, it is obvious that proof of absorption by one system does not necessarily exclude the other.

In a previous study the author (1927) reinvestigated the morphology of the lymphatic plexuses of the gall-bladder (confirming and extending Sudler's work), paying particular attention to the topographical relationships of the plexus subepithelialis. This plexus, which ramifies

in the subepithelial coat, is composed of minute, frequently anastomosing capillaries of quite constant calibre. A venous capillary plexus with more numerous interconnections is likewise situated in this layer, and resembles the pattern of lymph vessels so closely that difficulty may arise in differentiating these two systems. Nevertheless, two anatomical differences, which may later prove of possible physiological significance, permit of their differentiation. Firstly, the venous plexus is in closer proximity to the epithelial cells; and in many areas only one or two connective-tissue cells intervene; secondly, the venous plexus in its close relationship to the mucosa projects upward into the stroma of the mucosal folds. The lymphatic plexus, on the other hand, fails to dispatch any similar capillary structures to the folds, so that vessels analogous to the lacteals of the intestine are lacking.

This anatomical recapitulation has been presented because it bears on the description of the present experimental observations. In a brief preliminary report the author (1927) demonstrated that the gall-bladder mucosa is permeable to potassium ferrocyanide and iron-ammonium citrate in solution. The present study amplifies this observation. It will be shown that the venous system is the more important pathway of resorption, at least, for the experimental solution employed in this study. Further, it will be demonstrated that the epithelial cells of the gall-bladder mucosa exhibit a differential permeability. This may be explained by the fact that the cells do not present a uniform and constant morphology, but are structurally altered by their functional activity, and, moreover, that the cells of a given functional phase exhibit an increased permeability.

MATERIALS AND METHODS

Because of the exact knowledge of the lymphatic and venous plexuses in the gall-bladder of the cat, obtained by the writer (1927), this animal was chosen for the experimental study. A solution composed of equal parts of a one and one half per cent solution of potassium ferrocyanide and ferric ammonium citrate was selected for injection into the gall-bladder because, after precipitation as Prussian blue, it lends itself conveniently to histological examination.

A typical protocol was as follows: Food was withheld for twelve

hours previous to the experiment. A laparotomy was performed under ether anesthesia and the duodenum incised opposite the ampulla of Vater. A catheter was inserted and pushed up the common duct until its tip entered the cystic duct. The bile was expressed and the viscus irrigated with warm Locke's solution until the returning fluid was clear. The foreign solution, prepared just prior to the beginning of operation, was injected, until the gall-bladder became moderately filled (usually 3 to 4 cc. sufficed). The catheter was withdrawn, the duodenum closed with silk and the abdominal wall approximated.

After various periods of time, ranging from eight to eighty minutes, the animals were killed and the entire hepatic system removed; the catheter was reinserted, the viscus again washed out three times with warm Locke's solution and then filled with acid formalin. The entire specimen was then placed in acidified formalin for 24 hours and transferred to 10 per cent formalin. Thorough irrigation is necessary, both prior to and immediately after the conclusion of the experiment, to prevent the formation of an insoluble coagulum of bile and any postmortem diffusion of the residual unabsorbed solution. That the latter precaution is important was voiced by Hanzlik (1912) who, in experiments on the intestinal epithelium, found a considerable and uniform postmortem absorption. Failure to observe these two precautions in this study entirely defeats the experiment. Likewise, the slightest injury to the gall-bladder wall should be assiduously avoided, as any break in the mucosal surface, however small, permits a rapid diffusion of the solution into the subjacent stroma. Hence, any procedure in which the experimental solution is injected directly through the gall-bladder wall with a syringe or in which these precautions are not observed cannot be considered properly controlled. Ten animals were prepared and sacrificed at approximately ten-minute intervals following the introduction of the Prussian blue reagents into the lumen of the gall-bladder. Following complete fixation blocks of tissue were excised from several areas: the cystic duct, the fundus, the corpus, and the neck, from both the peritoneal and hepatic surfaces. The blocks were embedded in paraffin and longitudinal and transverse sections were cut. These were stained with carmalum which affords an excellent contrast to the precipitated Prussian blue. In two animals the foreign solution was injected into the hepatic ducts, instead of

FIG. 1

into the gall-bladder. Blocks of tissue from the liver were subsequently removed and prepared in a similar manner for microscopic examination.

RESULTS

Passage of the ferrocyanide-citrate solution through the mucous membrane occurred in each of the animals studied. This was evident by the appearance of the precipitated dye in the epithelial cells, in the tissue spaces of the subepithelial, as well as to a less extent, of the fibromuscular layer, and, in most instances, in the venous capillaries in the several coats of the organ. The Prussian blue could be traced, in the majority of preparations, from the capillary venous plexus of the mucosal folds into the venules of the subepithelial stroma, and, thence, outward through the muscularis into the veins of the subserous layer. The presence of the precipitated granules in the lymphatic capillaries or in the larger draining lymph trunks was only very rarely observed, even in those experiments representing the greatest resorption. In the few instances in which the Prussian blue was seen in lymphatics, it was usually limited to the larger vessels of the subserous coat and then appeared only in meager amounts.

Although resorption occurred in the entire series of experiments, regardless of the time factor, the maximal passage of the solution, as estimated by the histologically demonstrable amount of Prussian blue in the wall of the gall-bladder, took place between ten and thirty minutes after the injection of the solution into the viscus. The rate of absorption of the Prussian blue reagents undoubtedly varies in individual gall-bladders, but the data obtained coincide, nevertheless, with the speed of absorption reported in quantitative experiments (Igwa-

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FIG. 1. MICROSCOPIC SECTION OF MUCOSAL FOLD OF CAT'S GALL-BLADDER

The Prussian blue can be seen unequally distributed in the epithelial membrane; appearing as minute granules or as a diffuse blue coloration in the typical columnar cells, and as fine granular lines in the slender, less frequent cells. Note the cytoplasmic granules visible in the large columnar cells.

In the subepithelial stroma, the Prussian blue appears in the same form in the tissue spaces and in the lumina of the blood vascular capillaries. At the base of the fold three sections of a blood vascular capillary containing the solution can be seen. (Cat 6 of series. 5 micra—stained with carmalum. × 800.)

naga) and with the qualitative results of previous investigators who obtained positive tests in the lymph, the bile or the urine soon after injection. In the present series, there was a gradual increase in the amount of absorption up to a certain point in the experiment, after which no further augmentation occurs. In the gall-bladders of the animals killed in the first few minutes, a moderate degree of absorption had already occurred, as evidenced by the presence of the precipitated granules in the epithelial cells, and to a slight extent in the submucous stroma. The Prussian blue reaction attains its maximum in the animals sacrificed at about ten, twenty or thirty minutes and remains at this uniform level of passage throughout more prolonged experiments.

In the experiments in which the gall-bladder was subjected to the influence of the Prussian blue reagents over an extended period of time, an observation has been made which appears to be of significance in evaluating the results of studies upon resorption from this organ. In experiments prolonged beyond forty minutes, some areas of the mucosa contained groups of epithelial cells which were almost completely filled with the precipitated granules and which exhibited staining of the nucleus. In these areas massive passage of the solution had occurred into the surrounding tissue. The resulting picture of absorption was never so orderly as in the gall-bladders from experiments of shorter duration. The epithelial cells exhibiting nuclear staining in the prolonged experiments are undoubtedly injured or dead. According to the criteria observed in the supra-vital technique for staining living cells, any cell the nucleus of which takes up the stain is considered to be moribund or dead; the nucleus and cytoplasm become completely permeable and permit the unrestricted entrance of substances previously unadmitted. The same interpretation may account for the massive resorption obtained in this study in the gall-bladders exposed to the experimental solution for a relatively long period. The reason for the death of the cells appears to be definitely correlated with the duration of the experiment rather than with the concentration of the solution which remained constant. The concentration of the Prussian blue reagent in the present experiments is the same as that which has been used in other experimental studies without record of the nuclear staining observed here. A possible

explanation of the death of the epithelial cells under the circumstances described here may be that the epithelium absorbs water from the experimental solution much faster than the contained crystalloids, so that the salts become rapidly concentrated to such an extent that they exert a deleterious action upon the cells. This idea lacks experimental proof at the present time. Two other conditions will produce a similar artifact: either postmortem diffusion of any residual, unabsorbed dye which is not washed out immediately at the conclusion of the experiment, or the puncture of the thin vesicle wall with a syringe needle.

Hence in all resorption experiments on the gall-bladder extending beyond a short period, a microscopic examination of the tissue is necessary, as a control, to the qualitative analysis made on the bile, the urine, the blood or the lymph. Unless this is done, definitely to rule out possible absorption resulting from cell injury, the experimental data necessarily demand cautious interpretation. Such a histological check has never been undertaken in the previous investigations and the omission lays them all open to adverse criticism.

Therefore, in this study the gall-bladders in which the epithelial cells exhibited intranuclear coloration were disregarded, and the following presentation is limited to those experiments terminated in the ten to thirty minute interval. Absorption of the experimental salts commences immediately upon their introduction into the gall-bladder. During the first half hour period, moreover, their passage through the wall must be considered as physiologically normal in the absence of any demonstrable nuclear or cytoplasmic injury.

For convenience and clarity the ensuing description will follow the successive layers of the gall-bladder wall,—the epithelial cells of the mucosa, the tunica subepithelialis, the tunica muscularis, and the tunica subserosa, and finally the duct system.

Under low power of the compound microscope the most striking feature of the epithelial membrane of the gall-bladder of the cat is the presence of very narrow, elongated cells, irregularly interspersed among the typical columnar cells. In the experimental animals, the location of the elongated cells is marked by the presence of minute, bluish-green lines representing the precipitated Prussian blue, which is contained in these cells in much greater amount than in the more numerous columnar type (see fig. 1). So striking is this picture of thread-

like precipitation of the dye in these particular cells, that one is reminded of ostia or pores in the mucous membrane through which the Prussian blue is passing into the subjacent tissue. Unrestricted to any particular area, usually occurring singly but also in groups of two or three, these cells are seen at the summits and sides of the mucosal folds, as well as in the bases of the crypts. Numerically, in proportion to the predominant columnar cells, they are present in the approximate ratio of 1 to 15. Without the aid of the Prussian blue one can distinguish this cell in ordinary sections by its paucity of cytoplasm and by the dense, elongated and homogeneous nucleus which appears to fill almost the entire cell.

Further examination under the high power of the microscope discloses the fact that all of the precipitated granules are intracellular rather than intercellular. Moreover, the granular material is confined solely to the cytoplasm and is not precipitated in the nucleus. This observation speaks emphatically against the possibility that the cells are moribund or dead; on the contrary, they appear to be living and physiologically active. In height this cell is, as a rule, longer than the neighboring columnar cell and may project slightly beyond the free surface or into the connective-tissue stroma. The cytoplasm, if unoccupied by Prussian blue, is exceedingly meager in amount and nongranular. The spindle-shaped nucleus appears to fill almost the entire cell with the exception of a minute area of cytoplasm visible at either the apical or basal pole. Various amounts of Prussian blue are contained in these cells; in some, the cytoplasm is completely filled and obscures the nucleus; in others, very little if any of the dye is discernible. Nevertheless, on examining several sections from any one of the experiments, it is obvious that this cell-type is predominantly permeable to the solution.

The ordinary columnar cells, on the hand, appear only slightly permeable to the solution. Minute granules can be perceived in the cytoplasm, in both a supranuclear and infranuclear position (fig. 1). The paucity of dye found in these cells, in comparison to the amount found in the other type described above, is surprising; but it is beyond a doubt that the columnar cell permits the passage of the solution in small amounts. Much more of the solution is present in the subepithelial tunic than can be accounted for by passage through the slender

highly permeable cells, so that these cells, although sparingly colored, must, collectively, absorb considerable amounts of the foreign solution. The Prussian blue occurs in the columnar cell either in the form of infrequent, minute particles, or, as a pale blue color diffusely scattered throughout the cytoplasm. The structure of this cell corresponds to the usual columnar type, possessing abundant cytoplasm and containing many closely packed, rather large, clear supra-nuclear granules and a somewhat basally placed nucleus with delicate scattering of chromatin material.

In the subepithelial tunic, one finds the foreign material profusely distributed as a pale blue coloration in the tissue spaces; to a greater extent in the stroma of the mucosal folds, but also (increasingly with the duration of the experiment) in the deeper tissue, until finally it reaches the fibro-muscular layer. But more obvious is the fact that the previously described venous capillaries situated in the delicate stroma are clearly defined as pale blue dots or wavy lines dependent on the plane of section (fig. 1). They stand out sharply and can be followed in their course downward into the venous plexus which occupies a level just below the bases of the crypts. In these vessels, one can follow the foreign granular material as it continues outward through the muscularis, either in straight or diagonal vessels which emerge finally in the tunica subserosa, where they attain considerable size. Here the lumina of the veins are filled to various extents; some of them may contain none of the color at all. If present, the blue material is observed to be usually adjacent to the lining endothelial cells. Apparently, as one traces the granules into the venous channels of ever increasing size, the Prussian blue undergoes gradual dilution until it becomes invisible, as it cannot be followed in the venous vessels as they enter the hepatic tissue at the vesico-hepatic margin or into the area directly attached to the gall-bladder. Furthermore, it is not possible, even in the longest experiments, to demonstrate any of the precipitated Prussian blue in the portal vessels or in the sinusoidal capillaries of the liver tissue immediately adjacent to the gall-bladder.

The foreign granules are observed in only the smallest amounts in the lymphatic vessels; when found therein, they are usually confined to an occasional large channel of the plexus perimuscularis or plexus subserosa. Evidently the solution enters the lymphatics only in the

event that the resorbed fluid reached the tissue spaces in the deeper levels of the stroma. The bulk of the solution is immediately taken up on its entry into the musosal folds by the venous capillaries. In cross-sections of the hepato-duodenal ligament which includes the cystic duct and the large draining lymphatic trunks, the Prussian blue is never observed in the lymphatic vessels. If present, it is in such high dilution as to be beyond the range of visibility.

THE DUCT SYSTEM

In two of the experiments the ferrocyanide-citrate solution was injected under very moderate pressure up to the hepatic ducts. Examination of sections obtained from various areas of the liver showed that the solution traversed the larger divisions and eventually reached the smaller ramifications of the duct system; however, it was never found in the hepatic lobules. Passage of the solution through the hepatic duct wall likewise occurred, as in the case of the gall-bladder; this passage was evidenced by the presence of the Prussian blue, either in the form of minute particles, or as a diffuse bluish color in the peri-ductal connective tissue (fig. 2). In none of the sections examined could any cell be found comparable to the highly permeable one seen in the gall-bladder. A pale bluish color could be detected in the majority of columnar cells, and only an infrequent cell contained the Prussian blue in the form of minute granules. These cells therefore correspond closely, in their physiological reaction to this solution, to the columnar cells of the gall-bladder. One finds a relatively large number of the precipitated granules in the peri-ductal connective tissue, in spite of the fact that very little is observed histologically in actual transit through the epithelial cells. In the gall-bladder, on the other hand, owing to the presence of the slender, readily permeable cells, in addition to the columnar cells, the amount of resorption far exceeds that observed in the hepatic ducts. From the peri-ductal tissue spaces one can trace the solution in the form of minute granules extending radially from the duct. On one side of the duct the solution has reached and entered a branch of the adjacent portal vein; whereas, on the opposite side, it has penetrated the connective-tissue capsule of the liver and has surrounded eventually the individual liver cells, forming a bluish peri-cellular network two or three cells deep (fig. 2).

FIG. 2. CROSS-SECTION OF LIVER, REPRESENTING PASSAGE OF PRUSSIAN BLUE THROUGH WALL OF HEPATIC DUCT

To the right it has traversed Glisson's capsule and entered a branch of the portal vein; to the left it has pierced Glisson's capsule and forms a peri-cellular network about the hepatic cells. (Cat 6 of series. 5 micra—stained with carmalum. × 400.)

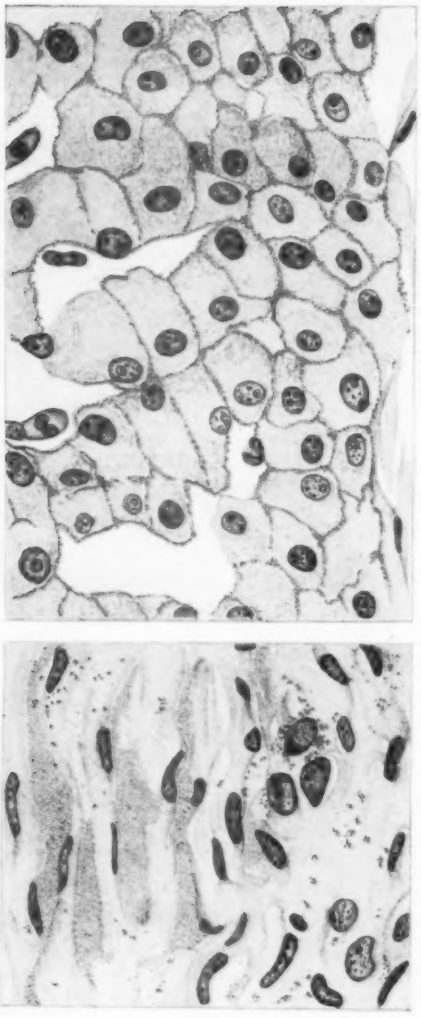

FIG. 3. AN ENLARGEMENT OF THE AREA OF LIVER PARENCHYMA DIRECTLY OPPOSITE THE BILE DUCT, INCLUDED IN FIGURE 2, SHOWING THE PERICELLULAR NETWORK OF PRUSSIAN BLUE

(Cat 6 of series. 5 micra—stained with carmalum. × 800)

FIG. 4. AN ENLARGEMENT OF GLISSON'S CAPSULE INTERVENING BETWEEN THE BILE DUCT AND PORTAL VEIN OF FIGURE 2 SHOWING THE FORM OF THE PRUSSIAN BLUE IN THE TISSUE SPACES

(Cat 6 of series. 5 micra—stained with carmalum. × 800)

Passage of the ferrocyanide-citrate solution through the epithelial cells of the smallest branches of the interlobular bile ducts does not occur, notwithstanding the presence of the solution in the lumina of these passages. Whether the solution was in contact with the cuboidal epithelium for too short a period could not be ascertained. But that the solution finds favorable resorptive conditions in the larger ducts cannot be doubted. On the contrary, the finding that the solution is not absorbed in those ducts which are only slightly smaller in diameter, suggests the question as to whether the smaller interlobular ducts are incapable of absorption. The observations of Kusnetzowsky lend some support to this suggestion. After injections of neutral fat reaching to even the smallest interlobular ducts, he found that there was no increase of intracellular lipoid in the cuboidal cells of the smaller ducts, whereas, in the columnar epithelium of the larger ducts, there was a considerable increase.

In the cystic and common ducts comparable conditions of permeability are found: the solution can be easily observed in the peri-ductal connective tissue in either the granular or the diffuse form, but never to the extent as seen in the gall-bladder. None of the narrow, elongated cells so prominent in the gall-bladder are found. The solution has apparently been absorbed by the columnar cells in small amount,—a process corresponding to the behavior of the same cell in the gall-bladder and in the hepatic ducts.

HISTOLOGICAL STUDY OF CYSTIC EPITHELIUM

The epithelial membrane of the gall-bladder has been considered heretofore as composed of uniform, simple, columnar cells. However the present investigation has shown that in the cat the mucosa is constituted of two morphologically different types of cells. In addition, these cells possess an unequal permeability to the ferrocyanide-citrate mixture. In order to make a more complete morphological differentiation sections were stained with hematoxylin and eosin, Mallory's and Van Gieson's stains, and muci-carmine. Likewise sections from corresponding areas of gall-bladders of several other mammals (sheep, armadillo, and human), were studied to discover whether the presence of more than one type of cell is a constant feature.

With the entire range of stains the two types of cells were readily

demonstrable in the mucosa of the cat's gall-bladder. Likewise in the other animals studied, as well as in the human gall-bladder, a similar differentiation of two types of cells could be made. With hematoxylin and eosin the nucleus of the slender cell appears dense and homogeneous, stains a bluish-black, and is narrow and elongated but of various lengths. Threads of amorphous-appearing, purplish-red cytoplasm surround the nucleus radially, and continue into either extremity of the cell. On the other hand, the typical columnar cells contain a bluish cytoplasm, more granular in form, especially in the supranuclear zone, where the granules attain a relatively large size. The periphery of each granule stains more deeply than the central portion, which remains comparatively clear. In some of the cells, small masses of these granules can be seen partially extruded at the free border. The nucleus of the columnar cell is large and oval and presents a fine, granular, chromatin substance. These cells heavily laden with granules are more numerous in the bases of the folds, and in the deepest crypts of the mucosa, where large areas of contiguous cells of this type can be seen. On the other hand, surmounting the mucosal folds, these cells present nuclei of variable size and staining intensity, and more striking variations in the number of cytoplasmic granules. One might group the cells in order of decreasing cytoplasm and declining nucleus, until one approaches the extreme stage,—the cell which was so abundantly filled with Prussian blue.

With Mallory's stain, a polychromatic reaction is obtained in all the cells; the nuclei stain orange, yellow or light blue, with the orange and yellow predominating in the cells of decreasing and intermediate size. The cytoplasm reacts similarly, that of the large, granular, columnar cells tending toward a light blue color. None of the epithelial cells exhibits the homogeneous blue of the connective-tissue stroma,—a finding which disproves an initial suspicion that the narrow, highly permeable cell might be of connective-tissue origin, intercalated amongst the columnar cells in the mucosa. With Van Gieson's stain, the epithelial membrane *en masse* stains a light yellow. Here, as well, there is no evidence that connective-tissue cells extend up and between the columnar cells.

With muci-carmine, the margins of the large cytoplasmic granules, present in the typical columnar cells, stain brownish red, whereas the

central portions remain practically clear or assume only a faint reddish tinge. Especially in the crypts and outpouchings of the mucosa, an intense staining reaction is obtained corresponding to the greater abundance of intracellular cytoplasmic granules, in contrast to the variable number of intracellular granules contained in those cells surmounting the mucosal folds. Here, irregular areas of cells stain rather deeply and others less intensely, and small masses of the supranuclear granules can be seen in part within the apical cell cytoplasm, and in part apparently extruded from the cell. The cytoplasm of the narrow cell, on the other hand, stains a homogeneous, pinkish-red or reveals no distinct color whatever. Between these two extreme types, one can trace the columnar cell gradually decreasing in size, with a corresponding diminution of granular cytoplasm, accompanied by a gradual shrinking and changing shape of the nucleus, until finally one reaches the slender cell in which there is a total absence of granules, the exhausted cytoplasm stains homogeneously, and the nucleus has become narrow and dense.

Thus, in examining many areas of the epithelial membrane it is observed that cells of intermediate size occur, and that all gradations in form and structure can be found ranging from the typical columnar cell, packed with round, apparently discrete, supranuclear granules to the slender cell with the dense nucleus and scanty cytoplasm.

The marked difference in permeability to the Prussian blue solution, as exhibited by these cells, was thought to be subject to two interpretations: first, that two cell types, morphologically and physiologically distinct, existed; or second, that the two types were merely extreme functional stages of the same cell—the columnar type representing a cell completely filled with accumulated, presecretory granules, in readiness to discharge its contents, and the narrow cell representing the same cell in a depleted state, at the end of excretion. The slender, functionally exhausted cell is apparently more permeable, at least to potassium ferrocyanide and iron ammonium citrate. Cells probably corresponding to the latter type have been described in several detailed histological studies of the gall-bladder, but the view has always been advanced that they assume this slender form from pressure exerted by adjacent cells. They are described as occurring more frequently in the crypts where the concavity of the membrane increases

the theoretical lateral pressure. If it were a question of external pressure associated with a partial folding of the mucous membrane, it would follow that these cells should be limited to the crypts; but this assumption is discredited by the occurrence of these cells scattered indiscriminately throughout the epithelial membrane,—at the apices or sides of the folds, as well as in the crypts. Furthermore, careful histological study of the cell content,—namely, the variable number of cytoplasmic granules and the nuclear alterations—argues against merely a pressure factor in their formation. Additional evidence of the variable morphology of the epithelium has been obtained in another way. Cells macerated in 33⅓ per cent alcohol reveal similar variations in the number of granules and differences in cell-size. It would seem most likely then that a given functional state of these mucus-secreting cells involves a specific structural picture. A similar histological description of the epithelial cells of the gall-bladder of several mammals has been given by Jurisch (1909). His interpretation coincides with the view expressed here,—namely, that accompanying the excretion of mucus, the epithelial cells present characteristic cytoplasmic changes equivalent to the alterations described above.

Mucus excretion by the epithelial cells of the vesica fellea has been debated by several histologists on the grounds that the characteristic staining reaction for mucus is not obtainable and that goblet cells are never observed in this membrane. The latter argument is immediately discredited by raising the question as to whether a cell in order to secrete mucus must necessarily assume a goblet shape. Present histological knowledge does not insist upon this structural transformation as an absolute essential antecedent stage preparatory to mucus secretion. In addition, presumptive evidence gained from several sources amply substantiates the view that mucus is elaborated by these cells. According to researches of Hammersten (1893) the mucus differs chemically from the mucin excreted by the intestinal epithelium and is of a nucleo-protein nature. This may explain the inability of some investigators to secure a sharp muci-carmine staining of the cytoplasmic granules. However, several workers (Shikinamai, 1910; Jurisch, 1909; and others) have reported obtaining a distinctive mucus reaction, particularly in the cells lining the depths of the crypts and in the outpouchings of the epithelium (Rokitansky-Aschoff

sinuses). Furthermore, more mucus is present in the cystic bile than can be accounted for by mere concentration, and in clinical cases where the cystic duct is obstructed by a stone, the gall-bladder is found containing essentially clear mucus. So the evidence obtained from several methods certainly indicates the production and excretion of mucus by these cells.

If the first suggested hypothesis were true, that two types of epithelial cells which are morphologically and physiologically distinct, occur in this membrane, one would then have to provide an explanation for the presence of the intermediate group of cells which present a partially depleted cytoplasm and a moderate reduction of nuclear size. Any attempted explanation of their presence involves logically the consideration of the two extreme types. The transitional character of the intermediate cells links the typical columnar cell with the typical slender cell; and, hence, strongly suggests the second view as the more probable explanation of the variable morphology of the epithelial cells, *e.g.*, the columnar cells and slender cells are merely functional stages of the same cell. (The view that these cells represent two distinct cells types or strains is not upheld.) The reason for the abrupt increase in permeability to the ferrocyanide-citrate solution exhibited by the narrow cell is not clear. It is merely apparent that, having fulfilled its secretory function and having become depleted of cytoplasm, the slender cell is physiologically exhausted, and at this moment becomes more highly permeable to the foreign solution.

DISCUSSION OF RESORPTIVE PROCESS

It might be well at this time to view critically the accumulated literature presented briefly in the introduction. Sound experimental proof has been advanced to justify the conclusion that the wall of the gall-bladder permits the passage of certain substances: first, from comparative chemical analyses alone, the passage of water and the inorganic salts; second, on histo-physiological grounds, the passage of neutral fat; and third, from chemico-physiological observations, the passage of various crystalloidal solutions. Nevertheless, with the exceptions of the experiments involving neutral fat, the investigators did not employ microscopic controls. As has been pointed out, the present experiments concerning the absorption of the Prussian blue reagents would have been entirely misleading without such control.

The demonstration by all investigators, with the exception of Mentzer, that neutral fat is resorbed by the lymphatics, whereas various crystalloids are resorbed for the most part by the veins, prompted Igwanaga to draw a close physiological parallelism between the epithelium of the entire extra-hepatic system and that of the intestine. He emphasises as a supporting argument their common embryological derivation. In all the experiments bearing on fat resorption, the experimental lipoid has been described as being transported in the lymphatics, and appears not only in the lymph capillaries of the mucosal folds, but also in lymph trunks lying in the deeper coats. This finding is open to adverse criticism on anatomical grounds, because lymphatic capillaries fail to ascend into the mucosal folds (Winkenwerder, '27). This criticism, however, does not affect the essential fact that the fat passes through the epithelial cells. But the present anatomical observations make it appear likely that the fat seen in vessels in the mucosal folds was actually within venous capillaries and not within lymphatics.

One may accept with considerable assurance the finding that experimentally introduced crystalloids (phenolsulphonethalein, methylene-blue, potassium iodide, indigo-carmine, phenolsulpho-cyanide) readily penetrate the biliary epithelium. The evidence advanced favors chiefly the venous system as the major pathway in the resorption. Many of the experiments have been merely qualitative, Igwanaga being the only worker to attempt a quantitative evaluation of the relative importance of the venous and lymphatic systems. His results coincide with the above interpretation. Moreover, the results of the present study indicate, by histological demonstration, that the venous system is the major pathway for the absorption of crystalloids. As emphasized by Heidenhain (1888), in relation to intestinal absorption, anatomical peculiarities, such as the closer proximity of the venous capillaries to the epithelial membrane, favor probable resorption by this system, rather than by the lymphatic system which lies in a less favorable position. Accepting this as probable, the rapid resorption, elicited by mechanical or bacterial inflammation, may be explained by the accompanying hyperaemia. That circulatory alterations may affect resorption finds experimental corroboration: vasoconstriction subsequent to nerve excitation (Reid, 1896), drugs

producing general vasoconstriction (adrenalin, Exner, 1903) and those producing a local effect (pituitrin, Rees, 1920) decrease resorption. Hyperaemia, on the other hand, accentuates the process (Hanzlik, 1912; Oschametsky, 1891).

Furthermore we are led to believe that, in addition to the crystalloids, colloidal solutions or suspensions, such as India ink, charcoal and particulate carmine likewise pass through the epithelial membrane; this belief is based on the grounds that the foreign substances were eventually recovered in the lymph nodes or in the surrounding tunics of the gall-bladder wall. Unqualified acceptance of these experiments would necessarily weaken the conception that the mucosa is a semi-permeable membrane and it would indicate a behavior decidedly different from that of other mucous membranes (*e.g.*, the mucosa of the intestine). Before accepting these observations, it must be first definitely shown that the passage of colloids or particles through the mucosa is not associated with a preliminary injury to the epithelium, resulting in an altered permeability of the mucosal coat. Substances of this nature might injure the mucosa purely mechanically or by their toxicity. Moreover, as has been shown in this paper, mere trauma to the gall-bladder incident to injection may be sufficient to produce abnormal phenomena of absorption. And further, it has been shown that even a relatively non-toxic solution such as potassium ferrocyanide and ferric ammonium citrate will, in thirty to forty minutes, injure the cells and be followed by massive resorption.

CONCLUSIONS

The epithelium of the entire extra-hepatic system, with the exception of that of the smallest bile ducts, is permeable to the crystalloids, —potassium ferrocyanide and ferric ammonium citrate.

In the gall-bladder, the epithelial cells apparently undergo structural changes in the discharge of their function, *viz.*, excretion of mucus.

The cells of a given functional phase, at the end of excretion, exhibit a greatly augmented permeability to the Prussian blue reagents.

The blood vascular and not the lymphatic system is the important resorptive pathway for the return, of the crystalloid solution employed, to the general circulation following its passage through the epithelial cells. This fact may be explained on anatomical grounds, in that the

blood capillaries are in closer proximity to the epithelial membrane, and are present in the form of capillary tufts in the mucosal folds.

The microscopic examination, shown to be necessary in such experimentation, for proper control and for exclusion of cell-injury, subjects many of the previous absorptive studies on the gall-bladder to serious question.

I wish to extend my appreciation to Mrs. N. T. Oates, of the Medical Art Department of the Johns Hopkins University, for the excellent illustrations and to Dr. G. B. Wislocki for criticism of the manuscript.

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GUANIDINE BASE CONCENTRATION IN BLOOD OF NORMAL INDIVIDUALS AND IN PATIENTS WITH LIVER INJURY[1]

READ ELLSWORTH

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Recently Minot and Cutler (1) have shown that dogs, fed on a meat diet low in calcium, are very susceptible to poisoning by carbon tetrachloride. Addition of calcium to the diet resulted in comparative tolerance to the drug, and administration of calcium salts, after intoxication had begun, usually brought about a cure. The poisoning was characterized by nervousness, gastro-intestinal irritation, and convulsions; followed by weakness, coma and death. There was a central necrosis of the liver, a more or less severe hypoglycaemia, and a retention of bilirubin and guanidine in the blood. Maintenance of comparable concentrations of guanidine in the blood by injection of that drug produced a similar intoxication, which in turn was relieved by the administration of calcium salts.

Previously, numerous investigators have reported in animals a retention of guanidine in the blood following parathyroidectomy [Paton and Findlay (2), Burns and Sharpe (3), Bayer, (4)]. These workers have also studied the intoxication produced in animals by the injection of guanidine salts. Watanabe, in a series of papers (5) (6) (7) (8), has observed the effect of guanidine administration upon the metabolism of rabbits and has noted the influence of the injection of calcium salts upon these animals after poisoning with guanidine.

Major and Weber (9, 10) found slight increases in the blood guanidine in certain patients with arterial hypertension. In many of their cases a chronic nephritis was present and the highest blood-guanidine figures reported by them occurred in patients with uraemia.

Burns and Sharpe (3) recorded an increase in blood-guanidine concentration in three children during active tetany.

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The work of Minot and Cutler (1), describing the association of diffuse liver damage, jaundice, and retention of guanidine in carbon tetrachloride poisoning in dogs, suggested to us the study of the blood guanidine in patients with liver injury. During the past year we have made blood-guanidine estimations in patients with a variety of diseases. The method used was that of Major and Weber (10).

METHOD OF DETERMINATION OF GUANIDINE

Fifteen cubic centimeters of whole blood are taken in oxalate and a protein-free filtrate made by the method of Folin-Wu. It is highly important that the filtrate should be protein-free. The usual precautions must be followed to ensure this: (1) Before filtering, the precipitation mixture is thoroughly shaken and allowed to stand at least 10 minutes. (2) The filter paper is moistened with the precipitation mixture and the first few cubic centimeters are re-filtered.

Eighty cubic centimeters of filtrate are taken and 0.5 cc. of 10 per cent NaOH added. One-half gram blood charcoal (Merck, purified by acid) is introduced and the flask shaken thoroughly. It is allowed to stand for 4 to 5 minutes and then filtered through a 9 cm. filter-paper. After draining 5 to 10 minutes, the paper is returned to the flask and 25 cc. acid alcohol (2 cc. NHCl to 100 cc. 95 per cent alcohol) added. This is shaken, allowed to stand over night (or at least 5 hours) and filtered. Twenty cubic centimeters of alcoholic filtrate are evaporated to dryness on a water-bath, care being taken not to char. The residue is taken up in 7 cc. of distilled water. Two cubic centimeters of the solution are taken off and set aside. One cubic centimeter nitroprusside reagent is added to the remaining 5 cc. It is centrifuged 2 minutes and read immediately in a colorimeter against a standard guanidine solution simultaneously prepared.

The standard guanidine is made immediately before the determination by diluting the stock guanidine solution 10 cc. to 1000 cc. with H_2O. The stock solution is made by dissolving 0.1542 gram guanidine carbonate in 100 cc. of 0.1 N HCl. It is preserved with chloroform. The standard solution contains 0.01 mgm. guanidine per cubic centimeter.

Standard tubes, set up with 2, 3, 4, 6, 8, and 10 cc. of standard guanidine solution, are all made up to 10 cc. with distilled H_2O. Two

cubic centimeters of nitroprusside reagent are then added to each tube.

The nitroprusside reagent consists of:

1 volume 10 per cent sodium nitroprusside
1 volume 10 per cent $K_3FeC_6N_6$
1 volume 10 per cent NaOH
9 volumes distilled H_2O

The nitroprusside reagent is mixed freshly for each series of determinations, but must stand at least half an hour before being used. By that time it will have faded to a light yellow color.

The colorimeter reading must be made within 5 minutes after the addition of the nitroprusside reagent to the unknown solution, because of the gradual development of some color due to creatinine. Also the reading must be made against the standard solution which the unknown matches most closely.

$$\frac{20}{R} \times 21.9 \times \frac{\text{mgm. in standard}}{2} = \text{guanidine bases in mgm. per 100 cc. of whole blood}$$

where R is the reading of the unknown in the colorimeter. Creatine and creatinine may be determined, if desired, upon the 2 cc. of extract set aside, and the appropriate correction for creatine made [*cf.* Major and Weber (10)]. The details of the chemistry underlying the method are described by Major and Weber (10) and by Minot and Cutler (1).

In the present paper only total guanidine base figures are given. Creatine corrections are not made. We have found the creatine determination on the final filtrate to be somewhat unreliable. Throughout the paper, blood guanidine concentration means the concentration of guanidine bases giving the immediate color reaction with the nitroprusside. All determinations in this paper were made with the same lot of charcoal. This charcoal gives an 80 per cent recovery of guanidine bases.

NORMAL VALUES

In order to ascertain what might be considered a significant increase in blood guanidine as determined by this method in our hands, we made

determinations upon a series of individuals who had neither hepatic nor renal disease. They were 25 convalescent patients, 16 males and 9 females, ranging in age from 13 to 57. The results are shown in Table I and are taken as our normal or control values. It is seen that the guanidine varies from 0.37 to 0.58 mgm. per 100 cc. of whole

TABLE I

Individuals without hepatic or renal disease

NUMBER	SEX	AGE	DIAGNOSIS	GUANIDINE PER 100 CC. BLOOD
				mgm.
1	M.	36	Duodenal ulcer	0.58
2	M.	57	Convalescent from slight myocardial insufficiency	0.53
3	F.	33	Convalescent from hyperthyroidism	0.48
4	M.	19	Convalescent from pneumonia	0.37
5	M.	13	Convalescent from myocardial insufficiency	0.46
6	F.	44	Peripheral neuritis	0.37
7	M.	50	Chronic sinusitis	0.41
8	M.	45	Convalescent from empyema	0.47
9	M.	44	Infected foot	0.40
10	M.	45	Psychoneurosis	0.55
11	M.	21	Convalescent from rheumatic fever	0.48
12	F.	54	Chronic bronchitis	0.47
13	F.	39	Convalescent from thyroid operation	0.58
14	F.	26	Convalescent from thyroid operation	0.40
15	M.	40	Chronic osteomyelitis	0.51
16	M.	30	No disease	0.45
17	M.	27	Convalescent from myocardial insufficiency	0.49
18	F.	33	Latent tetany, post-operative	0.43
19	M.	47	Carcinoma of stomach	0.50
20	F.	53	Intestinal obstruction	0.43
21	F.	20	Tuberculous pericarditis	0.41
22	M.	40	Convalescent from osteomyelitis	0.50
23	M.	45	Rheumatic heart disease, well compensated	0.37
24	F.	33	Ulcerative colitis	0.50
25	M.	48	Asthma	0.48

blood. The average finding is approximately 0.45 to 0.50 mgm., the upper limit approximately 0.60 mgm. of guanidine per 100 cc. blood. These results are somewhat higher than those reported by Major and Weber (10) in normal individuals. They are of the same magnitude as the values found by Minot and Cutler (1) in dogs.

BLOOD GUANIDINE AND JAUNDICE

The determination of blood guanidine in a series of patients with jaundice has yielded some interesting results. The cases have been divided into three groups; (1) those with icterus due to miscellaneous causes, (2) those with Laennec cirrhosis of the liver, (3) those with "arsphenamine jaundice." In the first group were five individuals, two of whom had gallstones; two, catarrhal jaundice, and one, hemolytic jaundice. The blood-guanidine estimations in these cases all fell within normal limits (Table II).

TABLE II

Jaundice, obstructive and hemolytic

NUMBER	DIAGNOSIS	VAN DEN BERGH	BILIRUBIN PER 100 CC. BLOOD	GUANIDINE PER 100 CC. BLOOD
			mgm.	mgm.
1	Cholelithiasis	Direct	5.6	0.55
2	Cholelithiasis			0.39
3	Catarrhal jaundice	Biphasic	4.7	0.45
4	Catarrhal jaundice	Direct	16	0.54
5	Hemolytic jaundice	Indirect	6.2	0.48

TABLE III

Laennec cirrhosis of the liver

NUMBER	DIAGNOSIS	VAN DEN BERGH	BILIRUBIN PER 100 CC. BLOOD	GUANIDINE PER 100 CC. BLOOD
			mgm.	mgm.
1	Laennec cirrhosis of liver*	Indirect	1.5	0.93
2	Laennec cirrhosis of liver	Indirect	5.2	0.91
3	Laennec cirrhosis of liver*	Indirect	1.5	0.76
4	Laennec cirrhosis of liver*	Indirect	1.2	0.92
5	Laennec cirrhosis of liver*	Indirect	0.25	0.73
6	? "Banti's Disease"	Indirect	Trace	0.86

* Diagnosis established at operation.

On the other hand, in the second group, consisting of five patients suffering from Laennec cirrhosis, four of whom had slight jaundice (1.5, 5.2, 1.5 and 1.2 mgm., respectively, blood bilirubin indirect van den Bergh), and one case diagnosed "Banti's disease," there was a moderate but definite increase in blood guanidine (Table III).

The third group of cases were those, in which jaundice occurred during the course of arsphenamine treatment. These are of especial

TABLE IV

Arsphenamine jaundice

CASE	DATE	CLINICAL CONDITION	VAN DEN BERGH	CALCIUM PER 100 CC. BLOOD	SUGAR PER 100 CC. BLOOD	BILIRUBIN PER 100 CC. BLOOD	GUANIDINE PER 100 CC. BLOOD
				mgm.	mgm.	mgm.	mgm.
1. J. K.	March 21	Mildly ill	Biphasic			8.6	0.78
2. O. G.	June 24		Biphasic			4.1	0.85
3. A. D.	May 2		Biphasic	9.7	98	9.2	0.90
	May 9		Biphasic			1.4	0.87
	June 3						0.53
4. M. S.	May 27		Biphasic		97	5.2	0.63
	May 29		Biphasic		85	6.2	
	June 1						0.93
	June 3		Biphasic	10.7	87	8.1	
	June 9						0.85
	June 12				95	1.4	
	June 14						0.51
5. E. T.	May 3			8.2			0.97
	May 6	Severely	Biphasic	8.6		8.3	0.90
	May 10	ill	Biphasic	10.4	96	12.3	
	May 14						0.73
	May 17		Biphasic			14.0	
	May 21		Biphasic	9.2	90	12.7	
	May 27		Biphasic	9.4	77	10.3	
	May 28						0.77
	June 12		Biphasic		80	2.4	0.63
	June 24	Marked	Biphasic			1.2	
	June 25	improvement					0.43
6. H. M.	May 17		Biphasic			11.8	0.67
	May 29		Direct	8.1		27	1.06
	June 4		Direct	10.2		40	1.25
	June 9						0.97
		Improvement begun					
	June 14		Direct			52	
	June 20	Marked	Biphasic			11.4	
	June 24	improvement	Biphasic			6.6	0.59

interest. There were, in all, six cases. Two (J. K. and O. G., Table IV) were seen only in the dispensary, as they refused admission to the wards. Three patients (A. D., M. S., E. T., Table IV) were kept in the wards and observed for a period of time. One (H. M.) was a private patient, and I am indebted to Dr. Joseph Earle Moore for the privilege of studying this case. Four of the cases (the two dispensary patients and two of the ward patients A. D., and M. S.) were considered mild cases of intoxication from arsphenamine. They were moderately jaundiced. The blood bilirubin varied from 4 to 9 mgm. per cent (van den Bergh). They complained of anorexia, some nausea, occasional vomiting and fullness in the epigastrium. The urine showed from a very slight trace to a trace of albumin, an occasional cast, large amounts of bilirubin. The spleen was usually palpable, the liver edge was at the costal margin or enlarged to one to two centimeters below.

In the two severe cases, in addition to the findings as outlined above, there was marked mental change. In one (E. T.) there was stupor, drowsiness and mental depression; in the other (H. M.) there was deep coma. In both cases there was a marked decrease in the size of the liver as determined by percussion. In one case (E. T.) leucine crystals were found in the urine. Both cases were regarded clinically as instances of acute hepatic necrosis ("acute yellow atrophy").

All six patients received a high-carbohydrate, low-protein diet. Fasting blood-sugar determinations were made from time to time as were estimations of blood guanidine and bilirubin. The results of the chemical examinations are given in Table IV.

DISCUSSION

In the 25 control individuals the blood guanidine varied from 0.37 to 0.58 mgm. per 100 cc. These results are of a slightly higher range than those found in normal persons by previous investigators (10).

The observations made upon five patients with jaundice due to miscellaneous causes showed that the blood guanidine fell within the normal limits; this would indicate that jaundice alone does not produce an increase in blood guanidine.

In the five patients with Laennec cirrhosis and in one with Banti's disease, who showed either slight icterus or none at all, the distinct

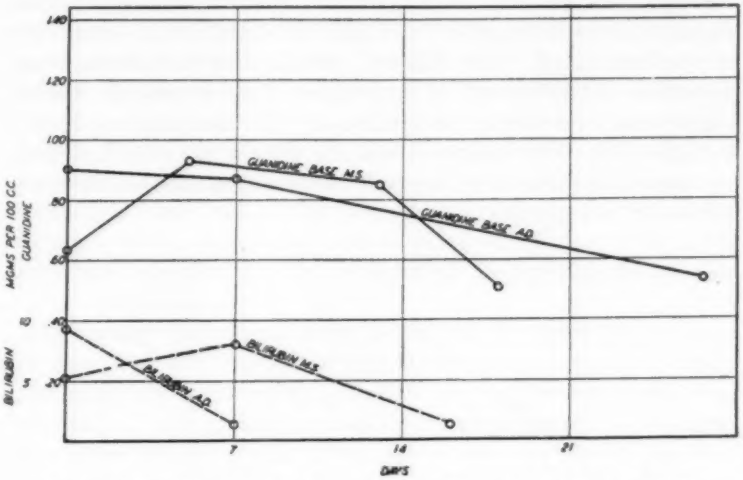

CHART 1. TWO MILD CASES ARSPHENAMINE JAUNDICE, A. D. AND M. S.

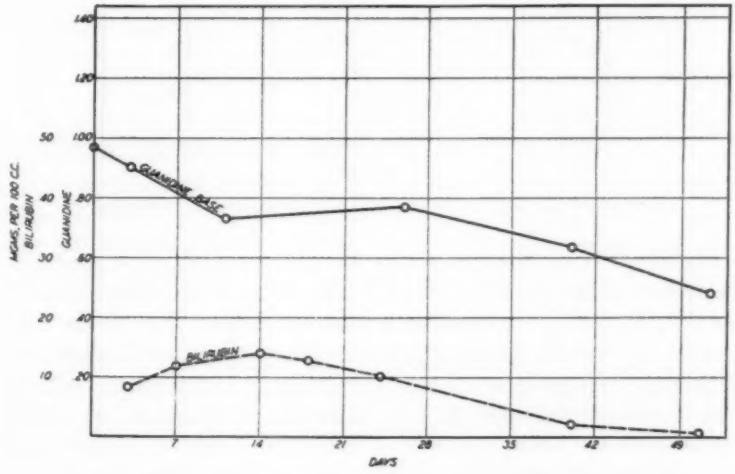

CHART 2. CASE E. T., SEVERE ARSPHENAMINE JAUNDICE

elevation of blood guanidine suggests the importance of diffuse liver damage as a factor in producing a high blood guanidine. One of the five patients (no. 1, Table III) was considered, at the time the blood guanidine was estimated, to be suffering from haemolytic icterus. Splenectomy was advised and performed. At the operation, definite cirrhosis of the liver was discovered. In three of the other four cases of cirrhosis an omentopexy was performed and the diagnosis definitely established at operation.

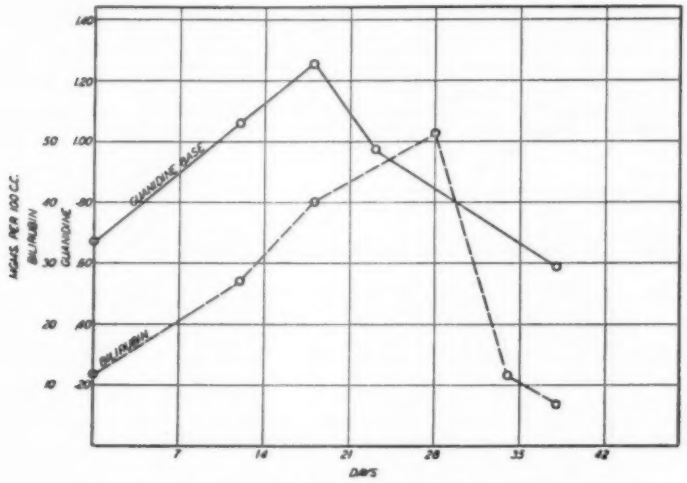

CHART 3. CASE H. M., SEVERE ARSPHENAMINE JAUNDICE

In all six cases of arsphenamine jaundice there was a conspicuous elevation of the blood guanidine (Table IV). In the severe cases (5 and 6, Table IV) the maximum value was higher than in the mild ones. In the four patients who were in the wards, two with severe and two with mild cases, and upon whom repeated observations could be made, the blood guanidine, elevated during the illness, fell to normal with recovery (Chartes 1, 2 and 3). Patients dying of arsphenamine poisoning characteristically show at autopsy diffuse liver damage of a type similar to that produced by Minot and Cutler with carbon tetrachloride in dogs. Here again, *i.e.*, in arsphenamine poisoning, diffuse liver damage is associated with high blood guanidine. In one case

(H. M.) the fall in blood guanidine occurred while the bilirubin was still increasing and preceded, by two days, the clinical indications of improvement in the condition of the patient. It is very difficult to estimate in these patients the degree of the liver damage and to judge at any particular time whether the outcome will be happy or fatal. Possibly the repeated estimations of blood guanidine may afford a means of following more accurately the progress of the intoxication.

Although these cases of arsphenamine poisoning showed slight albuminuria and occasionally casts, nothing comparable to an acute nephritis occurred and it is improbable that the guanidine retention is due to renal damage.

One fasting blood sugar determination (E. T., Table IV) was low (77 mgm. per 100 cc.). Otherwise there was no consistent hypoglycaemia such as occurred in the dogs poisoned with carbon tetrachloride (1). The glucose tolerance figures in the patient E. T., after 100 grams of glucose, are given below:

Time	*Blood sugar*
Fasting	96
½ hour after 100 grams of glucose	121
1 hour after 100 grams of glucose	131
2 hours after 100 grams of glucose	140
3 hours after 100 grams of glucose	143
4½ hours after 100 grams of glucose	105

These figures show an "increased tolerance" during the first hour and "decreased tolerance" later.

In one patient (H. M.) the three very high van den Bergh reactions were "direct." Three other van den Bergh determinations in this case and all estimations in the other five cases of arsphenamine jaundice were "biphasic."

SUMMARY AND CONCLUSIONS

A series of blood-guanidine determinations upon 25 individuals without hepatic or renal disease is reported. By the method employed the guanidine base values varied from 0.37 to 0.58 mgm. per 100 cc. of whole blood.

Guanidine estimations upon five patients with obstructive and hemolytic jaundice fell within the normal range.

In five cases of Laennec cirrhosis of the liver and one of "Banti's disease," there was a moderate but definite increase in blood guanidine.

In six cases of arsphenamine jaundice, there was a moderate to conspicuous increase in blood guanidine which fell to normal during recovery. The maximum concentration of blood guanidine was higher in the severe cases than in the mild ones. In one of the severe cases, the fall of blood guanidine just preceded the clinical signs of improvement.

These observations, upon patients with arsphenamine jaundice, in which diffuse liver damage is known to occur, and in patients with Laennec cirrhosis of the liver, together with the observations of Minot and Cutler in dogs, indicate that diffuse liver damage is an important factor in producing an increase in blood guanidine.

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